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Original Article

USP51 promotes deubiquitination and stabilization of ZEB1

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Abstract: ZEB1 is a transcription factor that induces epithelial-mesenchymal transition, tumor metastasis, and therapy resistance. ZEB1 protein is subject to ubiquitination and degradation, but the mechanism by which ZEB1 is stabilized in cells remains unclear. By screening a human deubiquitinase library, we identified USP51 as a deubiquitinase that binds, deubiquitinates, and stabilizes ZEB1. Depletion of USP51 in mesenchymal-like breast cancer cells led to downregulation of ZEB1 protein and mesenchymal markers, upregulation of E-cadherin, and inhibition of cell invasion. Conversely, overexpression of USP51 in epithelial cells resulted in upregulation of ZEB1 and mesenchymal markers. In addition, USP51 is able to regulate the expression of ZEB1 target genes. Importantly, USP51 is overexpressed in breast cancer patients and correlates with poor survival. Taken together, our findings suggest that USP51 is a ZEB1 deubiquitinase that may serve as an alternative pathway for targeting the cancer-promoting transcriptional factor ZEB1.

Keywords: ZEB1, deubiquitinase, USP51

Introduction

Metastasis remains a major challenge in cancer treatment and the leading cause of cancer-associated deaths [1, 2]. Despite the debate, emerging evidence from new genetically engineered mouse models and 3D models suggests that the epithelial-mesenchymal transition (EMT) and its reverse process, the mesenchymal-epithelial transition (MET), play critical roles in tumor metastasis [3-6]. During EMT, epithelial cells lose adherens junctions and tight junctions that keep them in contact with their neighboring cells [7]. Activation of EMT by several transcription factors induces migration, invasion, and metastatic dissemination of tumor cells [7]. ZEB1 is a master EMT-inducing transcription factor promoting tumor invasion, metastasis, and therapy resistance [8-13]. Aberrant expression of ZEB1 is associated with aggressive behavior, high tumor grade, treatment resistance, high metabolic plasticity, and metastasis in multiple cancer types [3, 9, 10, 13]. For instance, in breast cancer patients, high expression of ZEB1 in tumor cells was found in the aggressive subtype – triple-negative, basal-like breast cancer [14].

Posttranslational modifications regulate ZEB1 protein levels. Recently, the ubiquitin ligase Siah1/2 and the Skp1-Pam-Fbxo45 complex have been shown to promote ZEB1 ubiquitination and degradation [15, 16]. Moreover, we previously found that upon radiation exposure, ATM is rapidly activated, which phosphorylates and stabilizes ZEB1 protein, leading to upregulation of ZEB1 in radioresistant tumor cells derived from radiation treatment [10]. However, the mechanism by which ZEB1 protein is stabilized remains unclear.

Ubiquitination is a reversible process, and the removal of ubiquitin chains from proteins is mediated by deubiquitinating enzymes (DUBs, or deubiquitinases) [17]. DUBs are classified into six families, including the ubiquitin-specific

Table 1. List of primers used for RT-qPCR in this study

Gene	Forward	Reverse
ZEB1	AAAGATGATGAATGCGAGTC	TCCATTTTCATCATGACCAC
TWIST	CTAGATGTCATTGTTTCCAGAG	CCCTGTTTCTTTGAATTTGG
USP51	AAAAGAATGCTTTAGGTGGG	AAGGAATACTCCCTGACTTC
INADL/PATJ	CTGGACTAGGTTTTGGAATAG	ATCTCGATCTGCTAATCCTC
LOXL2	GATGTACAACTGCCACATAG	GACAGCTGGTTGTTTAAGAG
LLGL2	GTGATGAGAGAGTCCTGAAG	ATCAGTAGTAGTGTGTGCTC
CDH1	CCGAGAGCTACACGTTC	TCTTCAAAATTCACTCTGCC
SNAIL	CTCTAATCCAGAGTTTACCTTC	GACAGAGTCCCAGATGAG
VIM	GGAAACTAATCTGGATTCACTC	CATCTCTAGTTTCAACCGTC
MMP9	AAGGATGGGAAGTACTGG	GCCCAGAGAAGAAGAAAAG
MMP2	GTGATCTTGACCAGAATACC	GCCAATGATCCTGTATGTG
CDH2	ACATATGTGATGACCGTAAC	TTTTTCTCGATCAAGTCCAG
SLUG	CAGTGATTATTTCCCCGTATC	CCCCAAAGATGAGGAGTATC
ACTB	GACGACATGGAGAAAATCTG	ATGATCTGGGTCATCTTCTC
CRB3	CAAATACAGACCACTTCTGC	GGAGAAGACCACGATGATAG

protease (USP), ubiquitin carboxy-terminal hydrolase (UCH), ovarian tumor domain protease (OTU), Machado-Joseph disease protein domain protease (MJD), JAB1/MPN/Mov34 metallopeptidase (JAMM), and monocyte chemotactic protein-induced protein (MCPIP) families [18]. DUBs regulate both proteolytic degradation and non-proteolytic processes, such as DNA damage repair, cell cycle progression, gene transcription, and kinase activation [19, 20]. Mutation or aberrant expression of certain DUBs has been associated with various diseases, including cancer [21-23].

The deubiquitinase that regulates ZEB1 polyubiquitination and protein stability has not been reported yet. Here, we performed a DUB screen and identified USP51 as a ZEB1-binding DUB that promotes ZEB1 deubiquitination and stabilization. Previous studies have demonstrated that USP51 regulates cell differentiation [24], DNA damage response [25], and tumor growth [26]. In this study, we found that USP51 promotes cell invasion by stabilizing ZEB1, and that USP51 is upregulated in human breast cancer and correlates with poor survival.

Materials and methods

Cell culture

HEK293T, MDA-MB-231, and BT549 cell lines were from the American Type Culture Collection and cultured under conditions specified by the manufacturer. The LM2 subline of MDA-MB-231 cells was a gift from Dr. Xiang Zhang (Baylor College of Medicine) and the HEK293A cell line was a gift from Dr. Junjie Chen (MD Anderson Cancer Center); both cell lines were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C and 5% CO_2.

Plasmids and shRNAs

68 DUB ORFs were individually subcloned into an SFB (S-protein, FLAG tag, and streptavidin-binding peptide)-tagged expression vector (provided by Dr. Junjie Chen) using the Gateway system (Invitrogen) as described previously [27]. Full-length ZEB1 ORF and various deletion mutants were subcloned into a MYC-tagged expression vector using the Gateway system. The *mir-200b* promoter reporter construct (pGL3-321/+120) was from Addgene (plasmid number: 35540). GST-tagged USP51 was subcloned into the pDEST vector (Invitrogen). USP51 was subcloned into the pLenti6/V5-DEST Gateway vector (Thermo). The USP51-C372S mutant was generated using a QuikChange Site-Directed Mutagenesis Kit (Agilent). The primers used forgenerating the USP51 mutant are 5'-CCTGGACAATACAATTCATAAAAGAAGTGTTCCCAAGATTGATTAGC3' and 5'-GCTAATCAATCTTGGGAACACTTCTTTTATGAATTGTATTGTCCAGG3'. Human USP51 sh-RNA constructs were from sigma (sh-USP51#1, Clone ID: NM_201286.1-669s1c1, 5'-CCGGCCCAGAGACTAGGAAACGTAACTCGAGTTACGTTTCCTAGTCTCTGGGTTTTTG-3'; sh-USP51#2, Clone ID: NM_201286.1-1915s1c1, 5'-CCGGCCAACAGATTGTGTGCCCAATCTCGAGATTGGGCACACAATCTGTTGGTTTTTG-3'). The USP51 CRISPR-Cas9 plasmids were from Santa Cruz (sc-414805), containing a pool of 3 different gRNA plasmids (sc-414805A, sense: 5'-TCGAATGTGGCACAAGAGCC-3'; sc-414805B, sense: 5'-TGATCTACCAGCGTTTCGTT-3'; sc-414805C, sense: 5'-GTCTTCGAGACGTGAAGCCG3').

Lentiviral production and transduction

Lentivirus was produced and target cells were infected as described previously [28]. Lentiviral

supernatant was collected at 36, 60, and 84 hours after co-transfection of psPAX2, pMD2.G, and the shRNA- or ORF-containing vector into HEK293T cells, and was added to the target cells. 48 hours later, the infected cells were selected with drugs (10 μg/ml for blasticidin; 4 μg/ml for puromycin).

RNA purification and quantitative PCR (qPCR) analysis

Total RNA was extracted using TRIzol reagent (Invitrogen) and was then reverse transcribed with an iScript cDNA Synthesis Kit (Bio-Rad). Primers used in the qPCR experiments are listed in **Table 1**. cDNA was analyzed by qPCR using the SYBR Green Gene Expression Assays (Bio-Rad), and data were normalized to β-actin. Real-time PCR and data collection were performed on a CFX96 instrument (Bio-Rad). All qPCR reactions were performed in triplicate.

Immunoblotting

Cells were lysed in RIPA buffer supplemented with protease cocktail inhibitors and phosphatase inhibitors (Roche). The protein concentration was measured using the BCA Protein Assay (Bio-Rad). Western blot analysis was performed with precast gradient gels (Bio-Rad) using standard methods. Proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane (Bio-Rad). Membranes were blocked in 5% BSA in TBST for 1 hour at room temperature, and then incubated with the specific primary antibodies overnight at 4°C, washed in TBST, incubated with an HRP-conjugated secondary antibody for 1 hour at room temperature, and developed by the ECL reagent. The bands were visualized by chemiluminescence (Denville Scientific). The following antibodies were used: antibodies against USP51 (a gift from Sharon Dent, MD Anderson Cancer Center) [26], MYC (1:5,000, Santa Cruz, SC-40, clone 9E10), HSP90 (1:5,000, BD Biosciences, 610419, clone 68/Hsp90), HA (1:2,500, Santa Cruz, SC-7392, Clone F-7), ubiquitin (1:2,000, Santa Cruz, sc-8017), FLAG (1:5,000, Sigma, F3165, clone M2), E-cadherin (1:100, BD Biosciences, 610182), N-cadherin (1:100, BD Biosciences, 610921), Vimentin (1:1,000, Cell Signaling Technology, 5741P), β-actin (1:2,000, Santa Cruz, SC-47778), and GAPDH (1:1,000, Fisher Scientific, MA515738BTI).

In vitro binding assay

Bacterially purified MBP-ZEB1 was eluted with maltose and then incubated with glutathione beads (Amersham) conjugated with bacterially expressed GST-GFP or GST-USP51 at 4°C overnight. The glutathione beads was washed with NETN buffer (200 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.05% Nonidet P-40, 1 mM EDTA) and the bound proteins were eluted by boiling in Laemmli buffer and subjected to Western blot analysis.

Deubiquitination assay

HEK293T cells were co-transfected with ZEB1, DUBs, and HA-ubiquitin, and were treated with the proteasome inhibitor MG132 (10 μM) for 6 hours before lysis in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 1 mM EDTA). For denaturing, the lysates were heated for 5 minutes at 95°C in the presence of 1% SDS, followed by 10-fold dilution with RIPA buffer and sonication. ZEB1 was immunoprecipitated with anti-MYC beads and immunoblotted with the indicated antibodies.

Luciferase reporter assay

HEK293T cells at 70-80% confluence in 24-well plates were co-transfected with the DUBs, a firefly luciferase construct (the *mir-200b* promoter reporter construct, 25 ng), and the pRL-SV40 Renilla luciferase construct (0.5 ng). Two days after transfection, luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized with Renilla luciferase activity. Experiments were performed in triplicate.

Immunoprecipitation and pulldown assays

Cells were lysed in NETN buffer (200 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.05% Nonidet P-40, 1 mM EDTA) containing protease inhibitors (Roche). For pulldown of SFB-tagged proteins, cell lysates were incubated with S-protein beads for 2-4 hours at 4°C. For immunoprecipitation of protein complexes, cell lysates were pre-cleared with protein-A/G beads (Santa Cruz, SC-2003), incubated with the indicated antibody overnight at 4°C, and incubated with protein-A/G beads for 1-4 hours at 4°C. The

beads were washed three times with NETN buffer, and the bound proteins were eluted by boiling in Laemmli buffer and subjected to immunoblotting with the indicated antibodies.

Cell proliferation assay

2×10^3 cells in 100 μl culture medium were plated in 96-well plates and examined at 24, 48, 72, and 96 hours after plating. Cells were quantitated using the Vybrant® MTT Cell Proliferation Assay Kit according to the manufacturer's protocol. The absorbance at 540 nm was measured on a microplate reader (BioTek).

Colony formation assay

Equal numbers of control and USP51-knockdown LM2 cells were plated in 10-cm tissue culture dishes at a clonogenic density (500 cells/dish). Cells were cultured for 2 weeks. Colonies were stained with 0.05% crystal violet and counted.

Invasion assay

Matrigel Invasion Chambers (Corning) were warmed from -20°C to room temperature and rehydrated using serum-free DMEM mediun containg 0.1% BSA for 2 hours in a tissue culture incubator. 1×10^4 LM2 cells or 1×10^5 BT549 cells in 100 μl serum-free DMEM medium were plated in triplicate in the upper chamber. 500 μl medium containing 10% FBS was added to the lower chamber. After 18 hours of incubation in a humidified tissue culture incubator, the inserts were removed and washed with PBS several times. Cells on the upper surface of the membrane were removed with a cotton swab, and cells on the low surface of the membrane were fixed and stained with 0.1% crystal violet.

Immunohistochemical staining

Immunohistochemical staining was performed as previously described [28]. Paraffin-embedded breast tumor tissue microarrays (Biomax, BR243u) were deparaffinized, rehydrated, and subjected to a heat-induced epitope retrieval step in 0.01 M sodium citrate (pH 6.0). To block endogenous peroxidase activity, the sections were incubated with 0.3% (v/v) hydrogen peroxide in methanol for 30 minutes. The sections were then incubated with 0.5% Triton X-100 in PBS for 15 minutes and then 10% goat serum in PBS for 1 hour to prevent non-specific staining. Subsequently, samples were incubated with the antibody against USP51 (1:100, abcam, ab121147) or ZEB1 (1:100, Bethyl, A301-922A) for 1 hour at 37°C. After three washes in PBS, sections were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling Technology, 8114s). The color was developed using the DAB substrate Kit (Cell Signaling Technology, 8059s) according to the manufacturer's protocol. Counterstaining was carried out using hematoxylin. The sample was scored negative (0) when < 10% tumor cells showed expression. Postive scores (1 to 3) were based on the percentage of tumor cells showing positive staining, with 10%-25% defined as weak (1), 25%-50% defined as moderate (2), and > 50% defined as strong (3).

KM plotter analysis

Data were obtained from http://kmplot.com/analysis/. The *USP51* probe used for overall survival analysis was 237247_at. Auto-select best cutoff was used to define high versus low expression.

Statistical analysis

Unless otherwise noted, data are presented as mean ± SEM, and an unpaired two-tailed t-test was used to compare two groups of independent samples. Multiple comparisons were analyzed by one-way analysis of variance. The log-rank test was used to compare Kaplan-Meier survival curves. $P < 0.05$ was considered statistically significant. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

Results

USP51 binds ZEB1 and promotes its deubiquitination

To identify the DUB(s) responsible for ZEB1 deubiquitination, we conducted an ORF library screen for ZEB1-binding DUB(s) with a total of 68 human DUBs. SFB-tagged DUB was individually co-transfected with MYC-tagged ZEB1 into HEK293T cells and we pulled down the DUBs by S-protein beads. This initial screen identified 12 ZEB1-interacting DUBs (**Figure 1A**). In addition, endogenous ZEB1 protein in HEK293T cells could also be pulled down by these 12

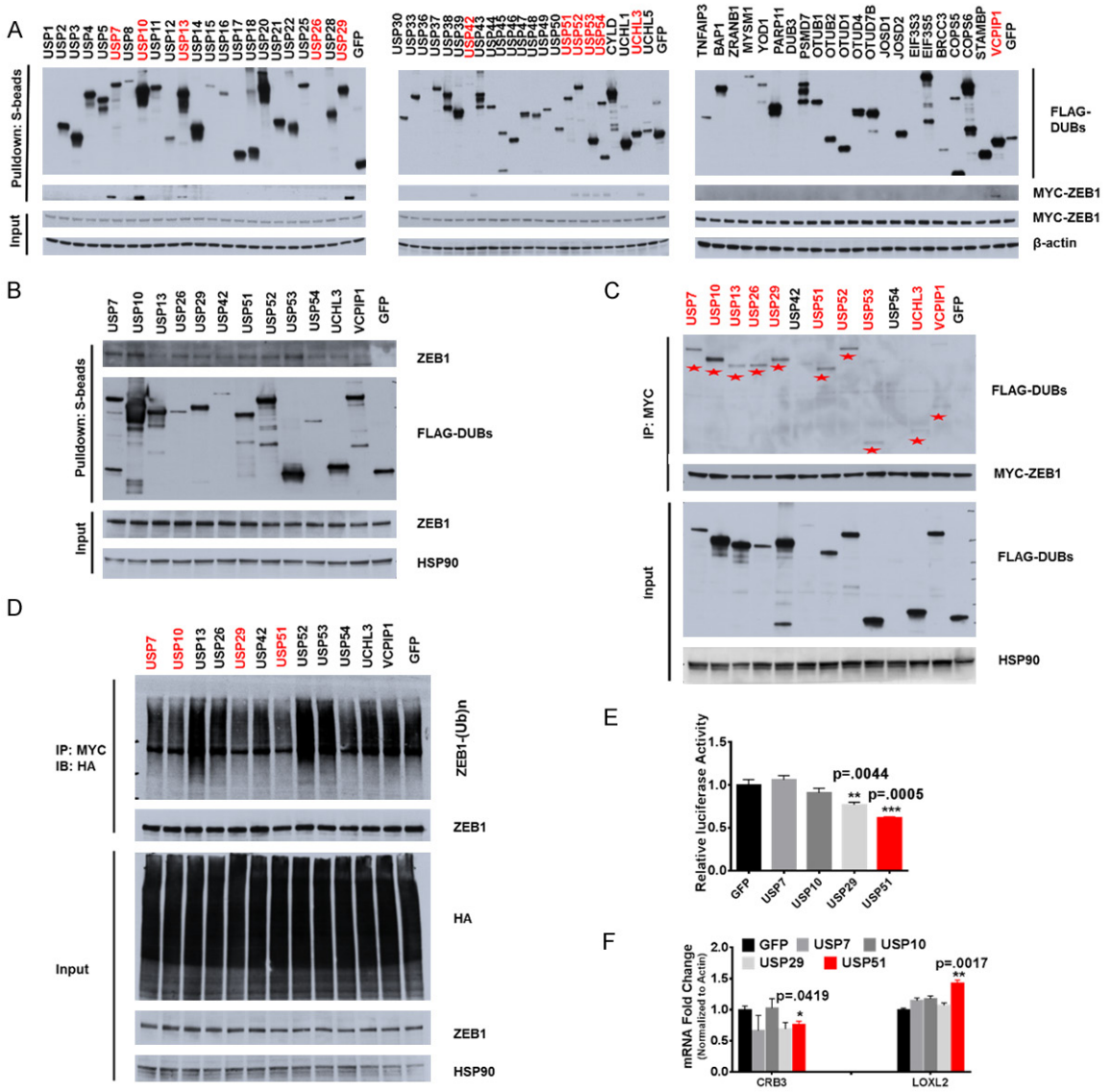

Figure 1. USP51 interacts with ZEB1 and promotes its deubiquitination. (A) SFB-tagged DUBs were co-transfected with MYC-ZEB1 into HEK293T cells, followed by pulldown with S-protein beads and immunoblotting with antibodies against FLAG and MYC. (B) 12 candidate DUBs were transfected into HEK293T cells, followed by pulldown with S-protein beads and immunoblotting with antibodies against ZEB1 and FLAG. (C) 12 candidate DUBs were co-transfected with MYC-ZEB1 into HEK293T cells, followed by pulldown with anti-MYC beads and immunoblotting with antibodies against FLAG and MYC. (D) HEK293T cells were co-transfected with MYC-ZEB1, HA-ubiquitin, and 12 candidate DUBs. After treatment with MG132 for 6 hours, cells were lysed, denatured, and subjected to pulldown with anti-MYC beads and immunoblotting with antibodies against HA and ZEB1. (E) Relative luciferase activity in HEK293T cells co-transfected with a mir-200b promoter reporter and the candidate DUBs. (F) qPCR of CRB3 and LOXL2 in HEK293T cells transfected with the candidate DUBs. Data in (E) and (F) are mean ± SEM. Statistical significance was determined by an unpaired t-test.

DUBs (**Figure 1B**). Reciprocally, when we pulled down MYC-tagged ZEB1, 10 out of 12 candidate DUBs were present in ZEB1 immunoprecipitates, further corroborating the physical association between ZEB1 and the identified DUBs (**Figure 1C**). In order to determine DUBs that deubiquitinate ZEB1, we performed a secondary screen of the 12 DUBs using an *in vivo* deubiquitination assay. To dissociate ZEB1 from its interacting proteins, we denatured the lysates before immunoprecipitating MYC-tagged ZEB1 protein from HEK293T cells co-transfected with MYC-ZEB1, HA-ubiquitin, and a candidate DUB. We found that four DUBs,

including USP7, USP10, USP29, and USP51, reduced the polyubiquitination of ZEB1 (**Figure 1D**).

Since ZEB1 represses the transcription of *mir-200b* and other target genes by binding to E-box sequences [29], a *mir-200b* promoter reporter assay was used to further validate DUBs that functionally regulate ZEB1. We found that, among four DUBs that decreased ZEB1 ubiquitination, USP51 inhibited the activity of the *mir-200b* reporter most significantly (**Figure 1E**). Although ZEB1 is a well-established transcriptional repressor, it can turn into a transcriptional activator by interacting with certain co-activators [30, 31]. RT-qPCR analysis revealed that USP51 was able to downregulate the ZEB1-repressed target gene, *CRB3,* and to upregulate the ZEB1-activated gene, *LOXL2* [32, 33] (**Figure 1F**), suggesting that USP51 is a positive regulator of ZEB1.

USP51 binds, deubiquitinates, and stabilizes ZEB1

To determine whether USP51 regulates USP51 protein or mRNA, we transfected LM2 (a lung-metastatic subline of the MDA-MB-231 cell line) and BT549 human breast cancer cells with USP51 siRNA. siRNA-mediated silencing of USP51 reduced the protein level of ZEB1 without decreasing the mRNA level of *ZEB1* in both cell lines (**Figure 2A**). Similarly, shRNA-mediated stable knockdown of USP51 in both LM2 and BT549 cells also downregulated ZEB1 protein (**Figure 2B**). Interestingly, CRISPR-Cas9-mediated knockout of USP51 in BT549 human breast cancer cells abrogated ZEB1 protein (**Figure 2C**). Next, we treated control and USP51-depleted LM2 cells with cycloheximide (CHX) to block protein synthesis and found that knockdown of USP51 markedly shortened the half-life of endogenous ZEB1 protein (**Figure 2D**).

Emerging evidence has shown that induction of EMT in tumor cells not only promotes metastasis, but also is associated with therapy resistance [34-36]. Our previous study demonstrated that ZEB1 protects breast cancer cells from irradiation by promoting homologous recombination-mediated DNA repair and the clearance of double-strand breaks, and that ATM-mediated phosphorylation is critical for radiation-induced stabilization and upregulation of ZEB1 protein [10]. Intriguingly, USP51 has been shown to deubiquitinate histones H2A and H2B and regulate DNA damage response [25, 26]. Since both ZEB1 and USP51 are involved in DNA damage repair, we asked whether USP51 is important for stabilizing ZEB1 upon radiation treatment. Consistent with our previous report [10], treatment of control LM2 cells with 10 Gy X-ray indeed upregulated ZEB1 protein. Interestingly, radiation also increased USP51 protein level (**Figure 2E**). Notably, knockdown of USP51 abolished radiation-induced upregulation of ZEB1 (**Figure 2E**), suggesting that USP51 is required for irradiation-induced ZEB1 stabilization.

To investigate whether USP51 directly regulates ZEB1, we performed *in vitro* binding assays and found that purified MBP-ZEB1 bound to purified GST-USP51 under cell-free conditions, demonstrating a direct interaction between ZEB1 and USP51 (**Figure 2F**). To determine the region of ZEB1 that associates with USP51, we generated five deletion mutants of ZEB1 [31]: ZEB1-Δ1 (amino acids 1-340), ZEB1-Δ2 (amino acids 1-781), ZEB1-Δ3 (amino acids 341-781), ZEB1-Δ4 (amino acids 341-1,124), and ZEB1-Δ5 (amino acids 782-1124). Co-immunoprecipitation experiments revealed that ZEB1's N-terminal region including the zinc finger domain mediated the interaction between ZEB1 and USP51 (**Figure 2G**). Interestingly, the N-terminal region of ZEB1 was also shown to bind the transcriptional co-factor p300 and p/caf [31, 37].

To further determine whether USP51 inhibits proteolytic ubiquitination of ZEB1, we transfected MYC-ZEB1 alone or together with SFB-USP51 into HEK293T cells, and treated the cells with 10 μM MG132 (a proteasome inhibitor) for 6 hours. We found that the polyubiquitination of ZEB1 was induced by MG132 treatment, which was reversed by overexpression of USP51 (**Figure 2H**). Moreover, expression of wild-type USP51, but not the catalytically inactive mutant (C372S) of USP51, reduced ZEB1 ubiquitination (**Figure 2I**), suggesting that the enzymatic activity of USP51 is required for USP51-mediated deubiquitination of ZEB1. Taken together, our data demonstrate that USP51 binds, deubiquitinates, and stabilizes ZEB1.

USP51 promotes breast cancer cell proliferation and invasion

In light of the role of ZEB1 in cancer, we asked whether USP51 is a cancer-promoting DUB. In

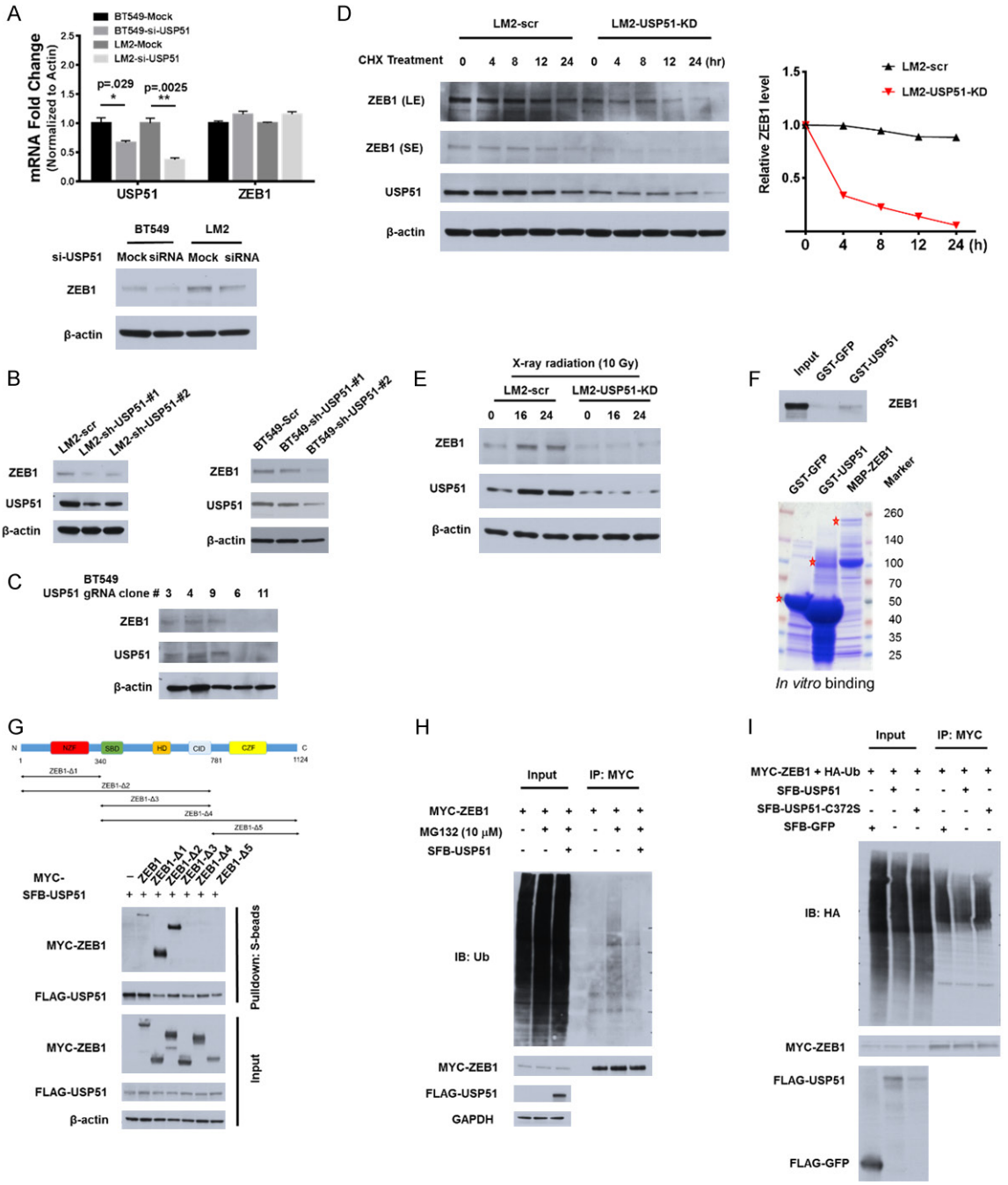

Figure 2. USP51 binds, deubiquitinates, and stabilizes ZEB1. A. qPCR (upper panel) and immunoblotting (lower panel) of ZEB1 in BT549 and LM2 cells transfected with USP51 siRNA. Data are mean ± SEM. Statistical significance was determined by an unpaired t-test. B. Immunoblotting of ZEB1, USP51, and β-actin in LM2 and BT549 cells transduced with USP51 shRNA. Scr: scramble control. C. Immunoblotting of ZEB1, USP51, and β-actin in USP51-knockout BT549 cells generated by CRISPR-Cas9. D. Left panel: scramble control (scr) and USP51-knockdown (USP51-KD) LM2 cells were treated with 50 μg/ml cycloheximide (CHX), harvested at indicated time points, and immunoblotted with antibodies against ZEB1, USP51, and β-actin. LE: long exposure; SE: short exposure. Right panel: quantification of ZEB1 protein levels (normalized to β-actin). E. Control and USP51-knockdown LM2 cells were treated with 10 Gy X-ray, harvested at indicated time points, and immunoblotted with antibodies against ZEB1, USP51, and β-actin. F. USP51 binds ZEB1 in vitro. Upper panel: GST-GFP or GST-USP51 was retained on glutathione-sepharose beads and incubated with MBP-ZEB1. The bound proteins were eluted by boiling in Laemmli buffer and immunoblotted with a ZEB1-specific antibody. Lower panel: recombinant GST-GFP, GST-USP51, and MBP-ZEB1 were purified from bacteria and analyzed by SDS-PAGE and Coomassie blue staining. G. Upper panel: schematic diagram of full-length ZEB1 and deletion mutants. NZF: N-terminal zinc finger domain; SBD: Smad-binding domain; HD: ho-

meodomain; CID: CtBP interaction domain; CZF: C-terminal zinc finger domain. Lower panel: MYC-tagged full-length ZEB1 and deletion mutants were co-transfected with SFB-USP51 into HEK293A cells. SFB-USP51 was pulled down with S-protein beads, followed by immunobloting with antibodies against MYC and FLAG. H. HEK293T cells were co-transfected with SFB-USP51 and MYC-ZEB1 and treated with MG132 (10 μM) for 6 hours. The whole-cell lysate was subjected to pulldown with anti-MYC beads and immunoblotting with antibodies against ubiquitin (Ub) and MYC. I. HEK293T cells were co-transfected MYC-ZEB1, HA-ubiquitin (Ub), and SFB-tagged GFP, USP51, or USP51-C372S, and treated with MG132 (10 μM) for 6 hours. The whole-cell lysate was subjected to pulldown with anti-MYC beads and immunoblotting with antibodies against HA and MYC.

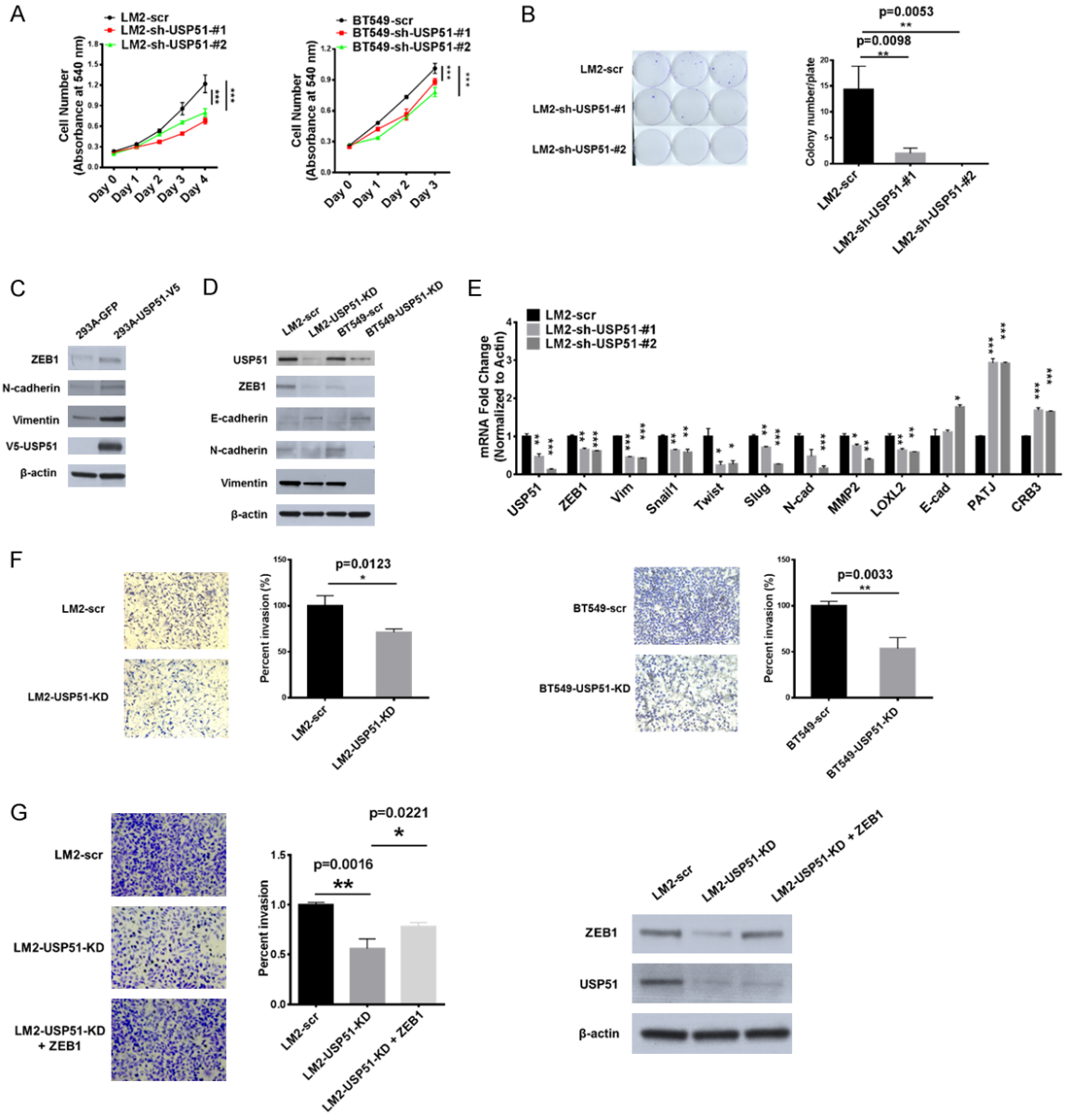

Figure 3. USP51 promotes breast cancer cell proliferation and invasion. (A) Growth curves of LM2 and BT549 cells transduced with USP51 shRNA. (B) Colony formation by LM2 cells transduced with USP51 shRNA. (C) Immunoblotting of ZEB1, N-cadherin, Vimentin, V5-USP51, and β-actin in HEK293A cells transduced with V5-tagged USP51. (D) Immunoblotting of USP51, ZEB1, E-cadherin, N-cadherin, Vimentin, and β-actin in scramble control (scr) and USP51-knockdown (USP51-KD) LM2 and BT549 cells. (E) qPCR of EMT markers and ZEB1 target genes in LM2 cells transduced with USP51 shRNA. (F) Invasion assays of scramble control (scr) and USP51-knockdown (USP51-KD) LM2 (left panel) and BT549 (right panel) cells. (G) Left and middle panels: invasion assays of USP51-knockdown LM2 cells with or without ectopic expression of ZEB1. Right panel: immunoblotting of ZEB1, USP51, and β-actin. Data in (A), (B), (E), (F), and (G) are mean ± SEM. Statistical significance was determined by an unpaired t-test.

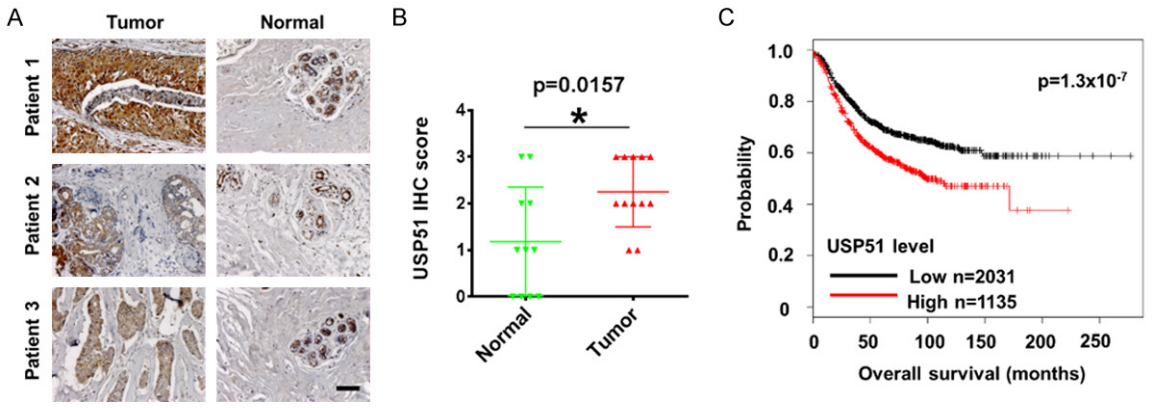

Figure 4. USP51 is upregulated in breast cancer. A. Immunohistichemical staining of USP51 in normal breast and breast carcinoma specimens. Brown staining indicates positive immunoreactivity. Scale bar = 100 μm. B. USP51 IHC scores in normal breast and breast carcinoma specimens. Data are mean ± SEM. Statistical significance was determined by an unpaired t-test. C. Kaplan-Meier curves of overall survival of breast cancer patients, stratified by USP51 expression levels. Data were obtained from http://kmplot.com/analysis/. Auto-select best cutoff was used in the analysis. Statistical significance was determined by a log-rank test.

agreement with a previous report [26], knockdown of USP51 reduced the proliferation rate of both LM2 and BT549 cells (**Figure 3A**). Moreover, USP51 depletion attenuated the colony-forming ability of LM2 cells (**Figure 3B**). ZEB1 is a master regulator of EMT, a developmental program that can be resurrected by cancer cells during metastatic progression [38]. We examined expression levels of EMT makers in stable cell lines with overexpression or knockdown of USP51. In the HEK293A epithelial cell line, ectopic expression of USP51 upregulated ZEB1 protein and the mesenchymal markers N-cadherin and Vimentin (**Figure 3C**). Conversely, knockdown of USP51 in two mesenchymal-like cell lines, LM2 and BT549, increased the protein level of the epithelial marker E-cadherin and decreased protein levels of ZEB1, N-cadherin, and Vimentin (**Figure 3D**). Furthermore, silencing USP51 in LM2 cells downregulated mRNA levels of multiple mesenchymal markers and upregulated the mRNA encoding E-cadherin (**Figure 3E**).

ZEB1 is a transcriptional repressor of epithelial genes, cell polarity factors, and stemness-inhibiting microRNAs [8, 9, 13]. However, when associated with certain coactivators (e.g. p300 and YAP), ZEB1 switches to a transcriptional activator of target genes known to stimulate tumor progression and metastasis [31, 32]. LOXL2, a promoter of tumor invasion, is transcriptionally activated by ZEB1 [32]. The tight junction of epithelial cells is crucial for the integrity of the epithelial barrier, and loss of tight junctions is a critical step for cancer cell dissemination and metastasis. PATJ and CRB3 are two tight junction proteins whose transcription is repressed by ZEB1 [33]. In the present study, knockdown of USP51 in LM2 cells decreased *LOXL2* mRNA level and increased *PATJ* and *CRB3* mRNA levels (**Figure 3E**), suggesting that the target genes of ZEB1 are regulated by USP51. Consistent with an MET phenotype (**Figure 3D** and **3E**), knockdown of USP51 in both LM2 and BT549 cells significantly reduced their invasiveness (**Figure 3F**), which could be partially reversed by ectopic expression of ZEB1 (**Figure 3G**). Taken together, these results suggest that USP51 maintains ZEB1 stability and activity and promotes invasion partly through ZEB1.

USP51 is upregulated in human breast cancer and correlates with poor survival

Since ZEB1 promotes EMT and metastasis, and since USP51 deubiquitinates and stabilizes ZEB1, we sought to determine whether USP51 is associated with tumor progression and clinical outcome. To this end, we analyzed the levels of USP51 protein in human breast tumor tissue microarrays, and found that USP51 was upregulated in human breast tumors compared with normal mammary tissues (**Figure 4A** and **4B**). Moreover, we performed Kaplan-Meier plotter analysis [39] and found that breast cancer patients with high USP51 expression in their tumors had worse overall survival than patients with low USP51 expression (**Figure**

4C). These data suggest that USP51 is upregulated in human breast cancer and correlates with poor clinical outcome.

Discussion

Metastasis remains the most common cause of cancer-related deaths. Identification and charaterization of metastasis genes and their mechanisms of action will lead to the develpoment of biomarkers and therapeutic targets of metastasis. The EMT-inducing transcription factor ZEB1 plays an essential role in cancer cell plasticity, metabolism, tumor recurrence, metastasis, and therapy resistance [3, 13]. Previous studies have demonstrated that ZEB1 protein is subject to proteolytic ubiquitination and can be stablized in certain conditions. In the present study, we identified USP51 as a deubiqutinase that promotes deubiquitination and stabilization of ZEB1. This finding is relevent in breast cancer, because USP51 is overexpressed in human breast tumors and because high expression of USP51 correlates with shorter overall survival in breast cancer patients. Moreover, several recent studies revealed that USP51 regulates DNA damage response and tumor growth. Here we show that USP51 promotes invasion through ZEB1. Whether USP51 promotes tumor metastasis, radioresistance, and drug resistance through ZEB1 warrants future investigation. Because DUBs are amenable to pharmacological inhibition by small-molecule inhibtors [40, 41], targeting USP51 may represent a new therapeutic strategy to deplete ZEB1 protein and overcome metastasis and therapy resistance.

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Disclosure of conflict of interest

None.

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References

[1] Chaffer CL and Weinberg RA. A perspective on cancer cell metastasis. Science 2011; 331: 1559-1564.

[2] Gupta GP and Massague J. Cancer metastasis: building a framework. Cell 2006; 127: 679-695.

[3] Krebs AM, Mitschke J, Lasierra Losada M, Schmalhofer O, Boerries M, Busch H, Boettcher M, Mougiakakos D, Reichardt W, Bronsert P, Brunton VG, Pilarsky C, Winkler TH, Brabletz S, Stemmler MP and Brabletz T. The EMT-activator Zeb1 is a key factor for cell plasticity and promotes metastasis in pancreatic cancer. Nat Cell Biol 2017; 19: 518-529.

[4] Nieto MA. Context-specific roles of EMT programmes in cancer cell dissemination. Nat Cell Biol 2017; 19: 416-418.

[5] Wei SC, Fattet L, Tsai JH, Guo Y, Pai VH, Majeski HE, Chen AC, Sah RL, Taylor SS, Engler AJ and Yang J. Matrix stiffness drives epithelial-mesenchymal transition and tumour metastasis through a TWIST1-G3BP2 mechanotransduction pathway. Nat Cell Biol 2015; 17: 678-688.

[6] Tsai JH, Donaher JL, Murphy DA, Chau S and Yang J. Spatiotemporal regulation of epithelial-mesenchymal transition is essential for squamous cell carcinoma metastasis. Cancer Cell 2012; 22: 725-736.

[7] Lamouille S, Xu J and Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. Nat Rev Mol Cell Biol 2014; 15: 178-196.

[8] Spaderna S, Schmalhofer O, Wahlbuhl M, Dimmler A, Bauer K, Sultan A, Hlubek F, Jung A, Strand D, Eger A, Kirchner T, Behrens J and Brabletz T. The transcriptional repressor ZEB1 promotes metastasis and loss of cell polarity in cancer. Cancer Res 2008; 68: 537-544.

[9] Schmalhofer O, Brabletz S and Brabletz T. E-cadherin, beta-catenin, and ZEB1 in malignant progression of cancer. Cancer Metastasis Rev 2009; 28: 151-166.

[10] Zhang P, Wei Y, Wang L, Debeb BG, Yuan Y, Zhang J, Yuan J, Wang M, Chen D, Sun Y, Woodward WA, Liu Y, Dean DC, Liang H, Hu Y, Ang KK, Hung MC, Chen J and Ma L. ATM-mediated stabilization of ZEB1 promotes DNA damage response and radioresistance through CHK1. Nat Cell Biol 2014; 16: 864-875.

[11] Thiery JP and Sleeman JP. Complex networks orchestrate epithelial-mesenchymal transitions. Nat Rev Mol Cell Biol 2006; 7: 131-142.
[12] Zheng H and Kang Y. Multilayer control of the EMT master regulators. Oncogene 2014; 33: 1755-1763.
[13] Zhang P, Sun Y and Ma L. ZEB1: at the crossroads of epithelial-mesenchymal transition, metastasis and therapy resistance. Cell Cycle 2015; 14: 481-487.
[14] Karihtala P, Auvinen P, Kauppila S, Haapasaari KM, Jukkola-Vuorinen A and Soini Y. Vimentin, zeb1 and Sip1 are up-regulated in triple-negative and basal-like breast cancers: association with an aggressive tumour phenotype. Breast Cancer Res Treat 2013; 138: 81-90.
[15] Chen A, Wong CS, Liu MC, House CM, Sceneay J, Bowtell DD, Thompson EW and Moller A. The ubiquitin ligase Siah is a novel regulator of Zeb1 in breast cancer. Oncotarget 2015; 6: 862-873.
[16] Xu M, Zhu C, Zhao X, Chen C, Zhang H, Yuan H, Deng R, Dou J, Wang Y, Huang J, Chen Q, Jiang B and Yu J. Atypical ubiquitin E3 ligase complex Skp1-Pam-Fbxo45 controls the core epithelial-to-mesenchymal transition-inducing transcription factors. Oncotarget 2015; 6: 979-994.
[17] Komander D, Clague MJ and Urbe S. Breaking the chains: structure and function of the deubiquitinases. Nat Rev Mol Cell Biol 2009; 10: 550-563.
[18] Xiao Z, Zhang P and Ma L. The role of deubiquitinases in breast cancer. Cancer Metastasis Rev 2016; 35: 589-600.
[19] Reyes-Turcu FE, Ventii KH and Wilkinson KD. Regulation and cellular roles of ubiquitin-specific deubiquitinating enzymes. Annu Rev Biochem 2009; 78: 363-397.
[20] Haglund K and Dikic I. Ubiquitylation and cell signaling. EMBO J 2005; 24: 3353-3359.
[21] Hussain S, Zhang Y and Galardy PJ. DUBs and cancer: the role of deubiquitinating enzymes as oncogenes, non-oncogenes and tumor suppressors. Cell Cycle 2009; 8: 1688-1697.
[22] Fraile JM, Quesada V, Rodriguez D, Freije JM and Lopez-Otin C. Deubiquitinases in cancer: new functions and therapeutic options. Oncogene 2012; 31: 2373-2388.
[23] Luise C, Capra M, Donzelli M, Mazzarol G, Jodice MG, Nuciforo P, Viale G, Di Fiore PP and Confalonieri S. An atlas of altered expression of deubiquitinating enzymes in human cancer. PLoS One 2011; 6: e15891.
[24] Kobayashi T, Iwamoto Y, Takashima K, Isomura A, Kosodo Y, Kawakami K, Nishioka T, Kaibuchi K and Kageyama R. Deubiquitinating enzymes regulate Hes1 stability and neuronal differentiation. FEBS J 2015; 282: 2411-2423.
[25] Wang Z, Zhang H, Liu J, Cheruiyot A, Lee JH, Ordog T, Lou Z, You Z and Zhang Z. USP51 deubiquitylates H2AK13,15ub and regulates DNA damage response. Genes Dev 2016; 30: 946-959.
[26] Atanassov BS, Mohan RD, Lan X, Kuang X, Lu Y, Lin K, McIvor E, Li W, Zhang Y, Florens L, Byrum SD, Mackintosh SG, Calhoun-Davis T, Koutelou E, Wang L, Tang DG, Tackett AJ, Washburn MP, Workman JL and Dent SY. ATXN7L3 and ENY2 coordinate activity of multiple H2B deubiquitinases important for cellular proliferation and tumor growth. Mol Cell 2016; 62: 558-571.
[27] Zhang J, Zhang P, Wei Y, Piao HL, Wang W, Maddika S, Wang M, Chen D, Sun Y, Hung MC, Chen J and Ma L. Deubiquitylation and stabilization of PTEN by USP13. Nat Cell Biol 2013; 15: 1486-1494.
[28] Zhou Z, Ji Z, Wang Y, Li J, Cao H, Zhu HH and Gao WQ. TRIM59 is up-regulated in gastric tumors, promoting ubiquitination and degradation of p53. Gastroenterology 2014; 147: 1043-1054.
[29] Burk U, Schubert J, Wellner U, Schmalhofer O, Vincan E, Spaderna S and Brabletz T. A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells. EMBO Rep 2008; 9: 582-589.
[30] Gheldof A, Hulpiau P, van Roy F, De Craene B and Berx G. Evolutionary functional analysis and molecular regulation of the ZEB transcription factors. Cell Mol Life Sci 2012; 69: 2527-2541.
[31] Lehmann W, Mossmann D, Kleemann J, Mock K, Meisinger C, Brummer T, Herr R, Brabletz S, Stemmler MP and Brabletz T. ZEB1 turns into a transcriptional activator by interacting with YAP1 in aggressive cancer types. Nat Commun 2016; 7: 10498.
[32] Peng DH, Ungewiss C, Tong P, Byers LA, Wang J, Canales JR, Villalobos PA, Uraoka N, Mino B, Behrens C, Wistuba, II, Han RI, Wanna CA, Fahrenholtz M, Grande-Allen KJ, Creighton CJ and Gibbons DL. ZEB1 induces LOXL2-mediated collagen stabilization and deposition in the extracellular matrix to drive lung cancer invasion and metastasis. Oncogene 2017; 36: 1925-1938.
[33] Aigner K, Dampier B, Descovich L, Mikula M, Sultan A, Schreiber M, Mikulits W, Brabletz T, Strand D, Obrist P, Sommergruber W, Schweifer N, Wernitznig A, Beug H, Foisner R and Eger A. The transcription factor ZEB1 (deltaEF1) promotes tumour cell dedifferentiation by repressing master regulators of epithelial polarity. Oncogene 2007; 26: 6979-6988.
[34] Martin TA and Jiang WG. Loss of tight junction barrier function and its role in cancer metasta-

sis. Biochim Biophys Acta 2009; 1788: 872-891.
[35] Singh A and Settleman J. EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. Oncogene 2010; 29: 4741-4751.
[36] Dave B, Mittal V, Tan NM and Chang JC. Epithelial-mesenchymal transition, cancer stem cells and treatment resistance. Breast Cancer Res 2012; 14: 202.
[37] Postigo AA, Depp JL, Taylor JJ and Kroll KL. Regulation of Smad signaling through a differential recruitment of coactivators and corepressors by ZEB proteins. EMBO J 2003; 22: 2453-2462.
[38] Yang J and Weinberg RA. Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. Dev Cell 2008; 14: 818-829.
[39] Gyorffy B, Lanczky A, Eklund AC, Denkert C, Budczies J, Li Q and Szallasi Z. An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1,809 patients. Breast Cancer Res Treat 2010; 123: 725-731.
[40] D'Arcy P, Wang X and Linder S. Deubiquitinase inhibition as a cancer therapeutic strategy. Pharmacol Ther 2015; 147: 32-54.
[41] Ndubaku C and Tsui V. Inhibiting the deubiquitinating enzymes (DUBs). J Med Chem 2015; 58: 1581-1595.